An Anti-HIV Peptide Construct Derived from the Cleavage Region of the Env Precursor Acts on Env Fusogenicity through the Presence of a Functional Cleavage Sequence

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A 22-amino-acid-long multibranched peptide construct (CLV) derived from the cleavage region (KIEPLGVAPTKAKRVRVQREKR*) of the human immunodeficiency virus (HIV) type-1 envelope precursor inhibits HIV infection (Virology, 1996, 223, 406±408). We attempted to characterize its activity for Env expressed via a recombinant vaccinia virus (rVV); gp160 cleavage was delayed, but not impaired, in the presence of CLV (10 μM), whereas neither Env production nor Env membrane expression was significantly altered. Through the synthesis of analogs, we concluded that the presence of a cleavage sequence was required for inhibition of syncytium formation by CLV in rVV-infected CD4+ cell cultures: indeed, a single amino acid residue substitution (R*>S) in the cleavage sites presented by CLV abolished its activity. Other analogs allowed us to further determine the region of CLV which mediates its activity. The ability of a radiolabeled CLV analog to enter cells was also shown. Altogether, these data strongly suggest that CLV acts on Env fusogenicity at least partially through interference with gp160 processing.© 1998 Academic Press

Key Words: HIV Env, gp160 cleavage, Env activity, anti-HIV peptides, protein processing.

INTRODUCTION

The surface glycoprotein gp120 and the transmembrane glycoprotein gp41 are cleavage products of the gp160 precursor and the components of the mature human immunodeficiency virus (HIV) type-1 envelope (Env) (Einfeld, 1996). gp120 and gp41 are non-covalently associated at the virus surface, where they mediate HIV binding to, and membrane fusion with, CD4+ cells, respectively (Moore et al., 1993; Einfeld, 1996).

The cleavage of gp160 into gp41 and gp120 is required for activation of the HIV envelope fusion function: mutation of gp160 cleavage sites impairs membrane fusion through precursor cleavage inhibition (McCune et al., 1988; Freed et al., 1989; Bosch and Pawlita, 1990). During its biosynthetic pathway, gp160 cleavage results in a trans Golgi compartment (Pfeiffer et al., 1997) from the action of proteases that have not been unequivocally identified: for some, furin activity is considered the candidate for Env cleavage but several works showed that subtilisin-like protein convertases also fulfill the expected criteria (Hallenberger et al., 1992, 1997; Morikawa et al., 1993; Onishi et al., 1994; Decroly et al., 1994, 1996, 1997; Gu et al., 1995; Inocencio et al., 1997; Einfeld, 1996).

Two potential conserved tryptic-like endoproteolytic cleavage sites represented by clusters of basic amino acid residues (aa) exist on gp160 (Bosch and Pawlita, 1990; Einfeld, 1996). A first site is defined by the sequence KAKRR (aa 505±509) and the other by the sequence REKR (aa 513±516). In vitro, furin cleaves more efficiently the REKR site when compared to the KAKRR sequence (Brakch et al., 1995). About 15% of mature recombinant and viral Env is cleaved at site 1 and does not organize in oligomers (Fenouillet and Gluckman, 1992) but it is clear that only the cleavage at site 2 leads to fusogenic gp41 species (Freed et al., 1989; Bosch and Pawlita, 1990); it is likely that the gp41 resulting from cleavage at site 1 is not functional because the additional polar aa sequence alters gp41 fusion peptide properties. Surprisingly, it was shown that cleavage of Env can occur when no dibasic sequence is present at the cleavage site, which raises questions about the specificity of the protease that cleaves (Dubay et al., 1995).

Various linear peptides (Callebaut et al., 1996; De Rossi et al., 1991; Neurath et al., 1992, 1995; Slepushkin et al., 1993) and synthetic multibranched peptides (SPCs) (Yahi et al., 1994; Benjouad et al., 1995; Barbouche et al., 1998a) derived from HIV Env interfere with HIV infectivity and syncytium formation in HIV-infected CD4+ cell cultures. The mode of action of these peptides remains unclear but they are supposed to interfere either with Env biosynthetic events or with post-CD4-binding events. Recently, we also reported preliminary data showing that a SPC (CLV; four branches of the KIEPLGVAPTKAKRVRVQREKR motif) derived from the sequence encompassing the cleavage region of gp160 inhibits HIV infectivity without cell toxicity (Sabatier et al., 1996). Based on this observation and because it was described that peptidyl chloromethylketones that possess the RXKR motif interfere with gp160 cleavage and inhibit HIV infectivity (Hai-
tium formation, and (iii) a control rVV lacking HIV

The gp160 cleavage into gp120 and gp41 in the presence or in the absence of CLV (10 μM) were incubated with a pool of HIV− sera to study the kinetics of Env membrane expression (Fenouillet et al., 1996, 1997): cells exhibited a similar level of Env at their surface irrespective of the presence of CLV (Fig. 1). Ab dilutions ranging from 1/50 to 1/50 000 led to similar data (not shown). Surprisingly but reproducibly (n = 3), the presence of CLV slightly increased labeling.

CLV delayed the cleavage of the gp160 precursor

The gp160 cleavage into gp120 and gp41 in the presence or in the absence of CLV (10 μM) was examined by pulse− chase labeling. The amount of immunopurified labeled Env was similar in all cases (Fig. 2A), in agreement with the data obtained by dot blot and ELISA. According to Fenouillet and Jones (1995) and Fenouillet et al. (1997), in the absence of CLV, gp120 was detected after a 30-min chase, and mature gp120Δ the increased MW of which reflects carbohydrate maturation (Fenouillet and Jones, 1995)Δ was detected after a 2-h chase. Similar data were obtained when cells were incubated with SPC C (not shown). Two hours postpulse, only a very faint band of gp160 was detected: it is likely that most of the uncleaved gp160 species was significantly degraded at that time and consequently was undetectable as a 160-kDa band after β-mercaptoethanol elution (as observed and discussed in Earl et al., 1991; Fenouillet and Jones, 1995). In the presence of CLV, gp160 appeared essentially uncleaved after a 30-min chase, whereas a significant cleavage was detected 2 h postpulse.
High-molecular-weight gp160 was still detected at this time. The migration of the Env species as double bands has been already observed (Fenouillet et al., 1997) in our discriminating SDS–PAGE/pulse–chase analysis conditions.

To further examine the ability of Env to be processed in the presence of CLV, a Western blot analysis of Env stored either in cell supernatant or in cell lysate 2 days postinfection was performed (Fig. 2B). The results supported the data presented in the pulse–chase study and those obtained by dot blot and ELISA as reported above: in the presence of CLV, a significant amount of cleaved gp41 and gp120 was detected into the cell pellet; after membrane expression, the gp120 subunit was efficiently secreted into the cell supernatant.

Thus, cleavage of Env was delayed, but not inhibited, in the presence of CLV.

CLV should present a functional cleavage site to be active

To determine the aa involved in CLV activity, we synthesized various analogs which were tested for their cell toxicity and ability to inhibit syncytium formation induced by rVV infection in CD4+ cell cultures, as described in Sabatier et al. (1996) (Table 2). Irrespective of their sequence and length, these analogs presented a cytotoxicity similar to that of CLV. The L analog which presents a R509/S516-S substitution in the cleavage sites failed to inhibit syncytium formation (Table 2). The fact that the S analog which presents a REKR motif without aa sequence downstream this site was inactive indicates that the REKR sequence including the R516 residue adjacent to the Lys core is not involved in CLV activity. In full contrast, the M analog which contains a cleavage region with aa upstream and downstream the cleavage site was as active as CLV, despite the deletion of a large part of the sequence upstream the first cleavage site. The ability of the various analogs to inhibit infection of C8166 cells by HIVMN was also examined: according to the data presented above, the L and the S analogs were found inactive (data not shown). These data indicate that CLV exhibited its anti-HIV activity through the presence of a functional cleavage sequence on the peptide construct.

CLV Y enters CEM cells

The ability of the active CLV Y analog to enter CEM cells (Fig. 3) was studied. We observed that CLV Y was able to rapidly bind cell membrane and enter cells (the

### Table 2

<table>
<thead>
<tr>
<th>SPC</th>
<th>Toxicity-free concentration (M)</th>
<th>Syncytium formation (IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLV:</td>
<td>[KIEPLGVAPTAKRRVVQREKR]K2-KβA</td>
<td>3·10⁻⁵</td>
</tr>
<tr>
<td>L:</td>
<td>[KIEPLGVAPTAKRRSVQREKR]K2-KβA</td>
<td>3·10⁻⁵</td>
</tr>
<tr>
<td>M:</td>
<td>[PTKAKRRVVQREKR]K2-KβA</td>
<td>3·10⁻⁵</td>
</tr>
<tr>
<td>S:</td>
<td>[VVQREKR]K2-KβA</td>
<td>3·10⁻⁵</td>
</tr>
<tr>
<td>C:</td>
<td>[GPGKTL]K2-K2-KβA</td>
<td>10⁻⁴</td>
</tr>
</tbody>
</table>

Note. [GPGKTL]K2-K2-KβA is a negative SPC control; a reduction of the syncytium score by 90% (IC90) relative to the negative control was considered to result from a significant inhibitory activity of the corresponding peptide.
amount of internalized CLV Y was calculated as described under Materials and Methods and in Barbouche et al. (1998b) as 'cell-associated radioactivity obtained when incubation was performed in the absence of NaNO$_3$' minus 'cell-associated radioactivity obtained when incubation was performed in the presence of NaNO$_3$'.

**DISCUSSION**

This work is the third to report the design and the characterization of peptide compounds derived from the endoproteolytic cleavage sequence of HIV Env. One of these works showed that decanoylated peptides derived from the gp160 cleavage region failed to inhibit in cell culture gp160 cleavage. Moreover, their important cell toxicity did not allow further development as anti-HIV therapeutic agents (Decroly et al., 1994).

In strong contrast, the pioneer work by Hallenberger et al. demonstrated that a set of peptide chloromethylketones (PCMK) which are derived from the gp160 cleavage region interfere with Env cleavage and hence its activation (Hallenberger et al., 1992). It is important to note that, in this work, the authors showed that an important cleavage of Env does exist (about 50% when Env was expressed by rVV in this study) in the presence of PCMK and that despite the expression of an important amount of cleaved Env, HIV infectivity is abolished.

We present here data which confirm and expand this latter work. Indeed, we showed that a 10 μM concentration of CLVD a peptide which encompasses a large sequence of the cleavage region of the Env precursor delayed, but did not prevent, gp160 processing, whereas it fully abolished syncytium formation. This compound is more potent than the PCMKs previously described (Hallenberger et al., 1992). The ability of a labeled CLV analog to enter cells is in agreement with such an intracellular effect. However, this interference with gp160 cleavage was not sufficient to alter substantially the amount of cleaved Env produced and expressed at the cell surface. The observed shedding into the cell supernatant of mature cleaved gp120 is another evidence that gp160 cleavage was not blocked by the treatment by CLV. Surprisingly, the presence of CLV slightly increased Env membrane expression, in agreement with the previous observation of Hallenberger et al. (1992), who observed that PCMKs slightly decrease shedding of gp120.

Because CLV altered at least the cleavage process during biosynthesis, we hypothesized that the presence of a cleavage substrate sequence on CLV was actually responsible for its interference with membrane fusion. To address this point, we synthesized various analogs of CLV on the basis of the data obtained in a previous study based on site-directed mutagenesis, which determined the aa required for Env cleavage (Bosch and Pawlita, 1990; Einfeld, 1996). First, we synthesized a CLV analog which presents a R &gt; S substitution in the cleavage sites. Such a mutation on env impairs Env cleavage (Bosch and Pawlita, 1990). Such an analog was inactive. Similarly, an analog which presents a REKR motif close to the uncharged Lys core lacked activity (The Lys core is uncharged as a result of the SPC synthesis). In full contrast, the M analog which presents a functional site 1 was active, despite the removal of several aa upstream to the functional cleavage site. From these data, we conclude that the sole presence of a functional cleavage region is responsible for CLV activity. This further argues in favor of a CLV-induced inhibition of syncytium formation through interferences with the enzymatic machinery involved in gp160 processing. Additional interferences of CLV with some steps of the membrane fusion process per se may act together with the interference with Env cleavage to completely impair membrane fusion. It is also possible that the presence of both cleaved and uncleaved Env within the same oligomers late in the intracellular routing or at the membrane disturbs the Env functions and structure to inactivate it. These hypotheses are under investigation to explain why CLV exhibits so potent an effect on Env functions despite its apparently slight effect on Env processing.

**MATERIALS AND METHODS**

Materials

D7324 (Aalto, Dublin, Ireland) is a polyclonal antibody (Ab) against gp120 C (aa 502–516); D7323 is against gp41 C (aa 845–860); 9305 and 9301 (DuPont de Nemours, Dreieich, Germany) are mouse monoclonal anti-gp120 Abs directed against aa 318±328 and aa 475±486, respectively. Chemical products were of analytical
grade (Sigma, St Louis, MO). Products for peptide synthesis were from Perkin-Elmer (Paris, France).

Synthesis of synthetic polymeric constructions

Chemical synthesis of SPCs was performed by the solid phase technique using Fmoc/t-butyliyl chemistry, as described by Yahi et al. (1994). The crude peptides were purified to homogeneity by C18 reversed-phase medium-pressure liquid chromatography (Barbouche et al., 1998a,b). The purity of the peptides was assessed by analytical C18 reversed-phase high-pressure liquid chromatography (Barbouche et al., 1998a,b). Fractions containing purified peptides were pooled and characterized by both aa analysis after acid hydrolysis and mass spectrometry. They were then tested in bioassays. SPCs are as follows: SPC C (a negative control): ([GPGLVTPLKAKVRVQREKR]4-K2-K-βA); SPC CLV: ([KIEPLGVAPTKAKRRVVQREKR]4-K2-K-βA); SPC CLV L (long): ([KIEPLGVAPTKAKRRVVQREKR]4-K2-K-βA); SPC CLV M (medium): ([PTKAKRRVVQREKR]4-K2-K-βA); SPC CLV S (short): ([VVQREKR]4-K2-K-βA); and SPC CLV Y (CLV analog used for radiolabeling): ([PTKAKRRVVQREKR]4-K2-K-Y).

Syncytium formation

Human lymphoid CD4+ CEM cells cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and 1% GlnD were infected in 12-well plates in the presence of various SPCs for 18 h with a recombinant vaccinia virus (rVV) expressing HIVLai Env (Kie`ny et al., 1988; VV9-1; 0.2 PFU/cell; a gift of Marie-Paule Kie`ny, against VV (Fenouillet et al., 1998a,b). The crude peptides were purified to homogeneity by C18 reversed-phase medium-pressure liquid chromatography (Barbouche et al., 1998a,b). Fractions containing purified peptides were pooled and characterized by both aa analysis after acid hydrolysis and mass spectrometry. They were then tested in bioassays. SPCs are as follows: SPC C (a negative control): ([GPGLKTLPK4-K2- K-βA); SPC CLV: ([KIEPLGVAPTKAKVRVQREKR]4-K2-K-βA); SPC CLV L (long): ([KIEPLGVAPTKAKRSVVQREKR]4- K2-K-βA); SPC CLV M (medium): ([PTKAKRRVVQREKR]4- K2-K-βA); SPC CLV S (short): ([VVQREKR]4-K2-K-βA); and SPC CLV Y (CLV analog used for radiolabeling): ([PTKAKRRVVQREKR]4-K2-K-Y).

Detection of Env membrane expression

Cells were infected with rVV for 1 or 2 days. After a wash in PBS, cells were treated with 2% paraformaldehyde. After 18 h, 3 · 10^6 cells were washed in PBS 2% casein (PBSC) and incubated for 2 h with a saturating concentration (1:100) of the pool of HIV-1 Abs (diluted 1/100 to detect Env species in the supernatant), (ii) with a mixture of 9305 and 9301 mAbs (diluted 1/50 to detect gp120 and gp160 in the lysates), or (iii) with D7323 (diluted 1/100 to detect gp41 and gp160 in the lysates). Incubations and washes were performed in PBS 0.5% casein, 0.5% Tween 20. Peroxidase-conjugated anti-Ads were then incubated for 1 h before staining with diaminobenzidine.
Internalization of an active radiolabeled CLV analog

The active CLV Y (5 μg) was labeled with 125I-labeled Na (300 μCi) in a 4 nM iodogen-coated tube for 30 min at 20°C (Barbouche et al., 1998a,b). Labeled CLV Y (sp act 50 μCi/μg) was purified on a PD10 column (Pharmacia). CEM cells were incubated with 125I-labeled CLV Y in cell culture medium for various times. Triplicates were performed (n = 2 experiments). For each incubation time, a triplicate was similarly incubated but the presence of 0.1% NaN3 to avoid endocytosis as reported in Barbouche et al. (1998a,b). Samples were then washed twice with PBSC, 0.1% NaN3 and cell-associated radioactivity was counted. Cell-associated radioactivity when incubation was performed in the presence of NaN3 represented membrane-associated CLV Y. Accordingly, 125I-labeled CLV Y present within the cells was calculated for each time point as follows: cell-associated radioactivity obtained when incubation was performed in the absence of NaN3 minus cell-associated radioactivity obtained when incubation was performed in the presence of NaN3 as reported by Barbouche et al. (1998b).

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